Isotopic homogeneity throughout the skin in small cetaceans

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RATIONALE: Isotope ratios from skin samples have been widely used to study cetacean trophic ecology. Usually, isotopic skin uniformity has been assumed, despite the heterogeneity of this tissue. This study aims to investigate 1) regional isotopic variation within the skin in cetaceans, and 2) isotopic variation among internal tissues.

METHODS: Stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios were measured in 11 skin positions in 10 common dolphins (*Delphinus delphis*) and 9 striped dolphins (*Stenella coeruleoalba*). Additionally, isotope ratios in the muscle, liver and kidney of both species were determined and compared with those from the skin and from all tissues combined. Signatures were determined by means of elemental analysis isotope ratio mass spectrometry (EA-IRMS).

RESULTS: In both species, no differences among isotope ratios of the skin positions were found. Moreover, the isotope ratios of skin were similar to those of muscle. In contrast, liver and kidney showed higher isotope ratios than muscle and skin.

CONCLUSIONS: Isotopic homogeneity within the skin suggests that the isotope ratios of a sample from a specific skin position can be considered representative of the ratios from the entire skin tissue in dolphins. This conclusion validates the results of previous stable isotope analyses in dolphins that used skin samples as representative of the whole skin tissue. Isotopic similarities or dissimilarities among tissues should be considered when analysing different tissues and comparing results from the same or different species.

INTRODUCTION

In recent decades, stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) have been extensively used to study diet, trophic relationships, migration and habitat use in marine mammals.[1–5] Carbon and nitrogen isotopes are used because the isotopic composition of consumers' tissues reflects that of their food resources.[6,7] The $\delta^{15}N$ value typically increases by 2-4‰ at each trophic level; therefore, it is an accurate indicator of a consumer's trophic position.[7–10] In contrast, $\delta^{13}C$ values are not used as trophic indicators in consumers because $\delta^{13}C$ value shows only a slight and typically non-consistent increase with trophic level (0-1‰). [11,12] However, $\delta^{13}C$ value is related to primary productivity and it is typically used to identify carbon sources along the food chain.[6] For example, in the marine environment, $\delta^{13}C$ values of benthic or inshore areas are higher than those in pelagic or offshore areas.[1,11,13]

In tissues, isotopic composition integrates information on assimilated diet over different timescales as body tissues differ in their turnover and metabolic rates.[14–16] Tissues with high isotopic turnover rates, such as plasma, reflect recent diet[17], whereas other tissues, such as bone, reflect long-term diet due to their lower turnover rates.[16,18] Due to this difference in turnover rate and also to differences in biochemical pathways, tissues do not acquire the isotopic composition of food in the same way, showing different $\delta^{13}C$ and $\delta^{15}N$ values.[19–22]

Among body tissues, the skin constitutes a suitable tissue for studying the diet of marine mammals as it can be easily collected with minimum harm from living animals using biopsy darts.[23–25] In bottlenose dolphins (*Tursiops truncatus*), the time required for the migration of epidermal cells from the basal lamina to the most external surface is approximately 73 days.[26] Furthermore, an experiment carried out by Browning et al.[27] with the same species under controlled diets determined that the half-lives of $\delta^{15}N$ and $\delta^{13}C$ values were 14-23 and 11-23 days, respectively. Taking this into account and considering that the time required for a complete integration of the new isotope ratios into a tissue is 2-3 isotopic half-lives, the skin isotope ratios reflects the integrated diet of the last two months.[28]

When collecting skin samples, it is usually assumed that the isotope ratios of a small tissue section are representative of the whole skin tissue.[29] However, the skin is not a homogeneous tissue. Depending on the location, the epidermis has a different thickness, being the ventral part the area typically showing the thickest layer.[30] Differences in the dermal papilla height[31] and colour, due to regional differences in the concentration of melanocytes[32,33], also occur within an individual. This heterogeneity may entail isotopic regional variations along the body skin, which, together with the difficulty of obtaining samples from the same region through remote darting (a method frequently used to obtain cetacean skin samples), may lead to bias in the results and to erroneous comparisons between studies.

The objectives of the present study were 1) to investigate regional variations in isotope ratios within the skin of cetaceans, and 2) to assess how nitrogen and carbon ratios vary among tissues.

With this purpose, $\delta^{15}N$ and $\delta^{13}C$ values were determined for 11 skin positions in the common dolphin and striped dolphin, and differences among them were evaluated. Variation among internal tissues and the skin of both species was also investigated. For that, $\delta^{15}N$ and $\delta^{13}C$ values were determined in these species for muscle, liver and kidney, three internal tissues with different turnover and discrimination rates.

MATERIAL AND METHODS

The samples used for the study were obtained from the biological tissue bank of the University of Barcelona (BMA Tissue Bank), where they were stored at -20°C. They had been collected from nine stranded striped dolphins in the Mediterranean Catalan coast between 2007 and 2009, and from ten common dolphins accidentally captured in the Northwest Spanish coast in 2001 and 2002. Similar number of males and females were considered for each species (Table 1). The 19 animals were freshly dead (carcass condition code II, following the carcass classification by Geraci & Lounsbury[34]).

These species were selected given that they are good representatives of small odontocetes and moreover, their sampling was easily carried out because they usually strand (striped dolphin) or are bycaught (common dolphin) in Spain.

For each animal, 11 skin positions were sampled in order to have the whole skin represented: four dorsal positions (1, 3, 6 and 9), three lateral positions (4, 7 and 10), and four ventral positions (2, 5, 8 and 11) (Figure 1). Liver, kidney and muscle were also sampled from all the animals.

Stable isotope analysis

Tissue samples were placed in Petri dishes, dried at 40°C for 24 h, and then powdered with a mortar and pestle. Since lipids may bias the analysis by decreasing $\delta^{13}C$ values, [6] they were removed from the samples by rinsing the powdered tissue several times with chloroform/methanol (2:1) solution.

After pretreatment, approximately 0.3 mg of each powdered sample was weighed into tin capsules and combusted at 900°C. Isotopic analyses were carried out by means of analyser/isotope ratio mass spectrometry (EA-IRMS) using a ThermoFinnigan Flash 1112 (CE Elantech, Lakewood, NJ, USA) elemental analyser that was coupled to a Delta C isotope ratio mass spectrometer via a ConFlo III interface (both from Thermo Finnigan, Bremen, Germany).

International isotope secondary standards of known $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios, namely, polyethylene (IAEA CH7; $\delta^{13}C$ =−31.8‰), sucrose (IAEA CH6; $\delta^{13}C$ =−10.4‰), ammonium sulphate (IAEA N1; $\delta^{15}N$ =+0.4‰ and IAEA N2; $\delta^{15}N$ =+20.3‰), potassium nitrate (USGS 34; $\delta^{15}N$ =−1,7‰), L-glutamic acid (USGS 40; $\delta^{15}N$ =−4,6‰; $\delta^{13}C$ = −26,2‰), and caffeine (IAEA 600; $\delta^{15}N$= 1,0‰; $\delta^{13}C$= −27,7‰), were used to calibrate the system and compensate for any drift over time. The reference materials used for the analysis were selected based on previous calibration experiments performed on the same type of samples to ensure an optimum range of reference values. All the reference materials used are distributed by the International Atomic Energy Agency (IAEA).

The stable isotope ratios were expressed in delta (δ) notation, where the relative variations of isotope ratios were expressed as permil (‰) deviations from the predefined international standards as follows:

$$\delta X = [(R_{sample}/R_{standard}) - 1]$$

where X is ^{13}C or ^{15}N, and R_{sample} and $R_{standard}$ are the heavy-to-light isotope ratios ($^{15}N/^{14}N$, $^{13}C/^{12}C$) in the sample and in the reference standards, respectively, certified by the IAEA. These standards are the Vienna Pee Dee Belemnite (V-PDB) standard and

atmospheric nitrogen for ^{13}C and ^{15}N, respectively. The accuracy of the $\delta^{13}C$ and $\delta^{15}N$ measurements was 0.1‰ and 0.3‰, respectively.

The C/N ratio for all samples varied between 2.92 and 3.78 (mean±S.D.= 3.16±0.61). These values (lower than 4) show that the lipid extraction process in the samples was effective.[35]

The analyses were conducted in the Centres Cientifics i Tecnològics of the University of Barcelona (CCiT-UB).

Data analysis

The normality and homoscedasticity of the data were tested using the Shapiro-Wilk ($n< 30$) and Levene's tests, respectively.

The isotope ratios of positions 5 and 9 in common dolphins and positions 4, 5, 8 and 10 in striped dolphins did not follow a normal distribution. Consequently, the Friedman test (a non-parametric test for related samples), followed by the post hoc Wilcoxon test and Bonferroni correction (to accommodate multiple comparisons), were used to detect differences in $\delta^{15}N$ and $\delta^{13}C$ values among the 11 skin positions.

The isotope ratios for skin, muscle, liver and kidney followed a normal distribution and presented homogeneity of variances. To compare the skin with the other tissues, the mean of $\delta^{13}C$ and $\delta^{15}N$ values of the 11 skin positions was calculated. The one-way repeated measures ANOVA, followed by post hoc pairwaise comparisons adjusted for multiple comparisons (Bonferroni), was used to detect differences of $\delta^{15}N$ and $\delta^{13}C$ values among tissues.

All the statistical analyses were performed using the statistical package SPSS 15 (SPSS Inc., Chicago, IL, USA).

RESULTS

The mean stable isotope and standard deviation (S.D.) ratios for each tissue and each position within the skin of common and striped dolphins are presented in Table 2.

Isotope ratios were more scattered in common than striped dolphins, either in skin positions or tissue types (Figures 2 and 3). $\delta^{15}N$ values were higher for common (11-15‰) than for striped dolphins (9-13‰) (Figures 2 a, b and 3 a, b).

In the current study, the sample size was small, and thus the power to detect differences was lower than desired for the comparison analyses.

Comparison of the isotope ratios among the 11 skin positions

Differences in $\delta^{15}N$ value: No significant difference in $\delta^{15}N$ value was detected among the different skin positions for common ($p = 0.751$) or striped ($p = 0.137$) dolphins (Figure 2 a, b).

Differences in $\delta^{13}C$ value: Both species showed significant differences among skin positions for $\delta^{13}C$ value (common dolphin $p = 0.001$, striped dolphin $p = 0.001$) (Figure 2 c, d). However, after Bonferroni correction, no significant differences were detected among skin positions.

Comparison of the isotope ratios among tissues

Differences in $\delta^{15}N$ value: Significant differences in $\delta^{15}N$ values were detected among the different tissues for both common ($p = 0.000$) and striped dolphins ($p = 0.002$) (Figure 3 a, b). In both species, the isotope ratios of liver and kidney were significantly higher than those of skin and muscle ($p < 0.008$), but no significant differences were found between liver and kidney or between skin and muscle (Figure 3).

Differences in $\delta^{13}C$ value: No significant differences in $\delta^{13}C$ values were found among the different tissues of common dolphins ($p = 0.453$) (Figure 3 c). However, in striped dolphins, $\delta^{13}C$ values in the kidney were significantly higher than in the skin ($p = 0.012$) (Figure 3 d). Although differences were not significant in most of the cases, $\delta^{13}C$ values in both species were higher for liver and kidney than for skin and muscle.

DISCUSSION

Striped and common dolphins are worldwide cetacean species considered as generalist feeders. Striped dolphins in the Western Mediterranean are known to feed on a huge variety of preys living in oceanic, pelagic or bathypelagic zones.[36] Common dolphins, inhabiting Atlantic Spanish coasts, feed mainly on blue whiting, sardine and mackerel.[37] A study carried out by Giménez et al.[25] in the South Western Mediterranean Sea, suggested that common dolphins might be more generalists than striped dolphins because they showed a wider isotopic niche. This fact might explain the higher dispersion of the isotope data in our study for common compared to striped dolphins, as the first species seems to consume a wider diversity of preys isotopically different.

On the other hand, despite striped dolphins normally exploit a slightly higher trophic level than common dolphins[25,38], $\delta^{15}N$ values in common dolphin tissues were higher than those of striped dolphin. This fact is explained by the higher $\delta^{15}N$ baseline in the Atlantic waters compared to the Mediterranean Sea.[39]

Comparison of isotope ratios among the 11 skin positions

The current study found homogeneity in both carbon and nitrogen isotope ratios in the skin throughout the body in both dolphin species. Only two previous studies have analysed isotopic differences among different skin positions: one performed in cetaceans[40] and the other in pinnipeds[41]. Both of these studies evaluated a lower number of positions and considered a lower number of individuals per specie than the current study. In the study of Williams et al.[40] the isotope ratios of two bottlenose dolphins were compared among four skin positions: the dorsal fin, a mid-thoracic site parallel with the dorsal fin, the leading edge of the fluke, and the dorsal surface of the fluke. Similarly, Todd et al.[41] analysed six skin and muscle positions, i.e., the neck, axilla, maximal girth, flank and dorsally above the pelvic girdle and hips, in three pinniped species: Steller sea lion (*Eumetopias jubatus,* n=5), California sea lion (*Zalophus californianus,* n=6) and harbour seal (*Phoca vitulina,* n=7). Both studies, reported no significant differences in isotopic composition ($\delta^{13}C$ and $\delta^{15}N$ values) among the different skin positions analysed, in agreement with the results obtained in the current study.

Altogether, these results suggest that there is sufficient homogeneity within the skin to consider the isotope ratios of a sample from a specific skin position as representative of the whole skin in dolphins. This finding is especially interesting as many cetacean skin

samples are obtained in nature through biopsy darting, where it is extremely challenging to obtain samples from the target areas: the dorsal fin or a nearby area.

Moreover, current results validate both: previous studies that chose a skin sample as a representative of the whole skin for stable isotope analysis, and comparisons between studies in which different skin regions were sampled, especially for common and striped dolphins, in which many studies, performed in different geographical areas, have focused for stable isotope analysis.[25,38,42–44]

Comparison of isotope ratios among tissues

When employing tissue isotope ratios to reconstruct diets and study trophic relationships, it should be taken into account that different tissue types present different isotope ratios due to differences in tissue composition and function. Thus, differences in protein composition and metabolic routing of dietary components among tissues might yield dissimilar isotopic compositions.[1,10,14,16] For example, the $\delta^{15}N$ and $\delta^{13}C$ values of different amino acids in a single tissue may vary more than 15%.[45]

In the present study, isotope ratios in both species showed important differences between tissues. However, skin and muscle did not show significant differences in either $\delta^{13}C$ or $\delta^{15}N$ values for both species. Similar findings have been reported for fin whales (*Balaenoptera physalus*)[45], humpback whales *(Megaptera novaeangliae)* [46], beluga whales *(Delphinapterus leucas)*[47] and pinnipeds (California sea lions and harbour seals)[41]. In contrast to these results, Meissner et al.[43] found that striped dolphins in the French Mediterranean coast presented $\delta^{15}N$ values significantly higher in the skin than in the muscle, which they attributed to the higher fractionation of skin compared to muscle. Similar findings have been reported for $\delta^{15}N$ values in other species, like grey *(Eschrichtius robustus*) and bowhead (*Balaena mysticetus*) whales, whereas $\delta^{13}C$ values were similar in both tissues. [47]In other cases, both stable isotopes differed significantly between skin and muscle, as it happened in pilot whales (*Globicephala melas*).[48]

Thus, further studies should be performed to determine whether skin and muscle can be used interchangeably in stable isotope analysis. If isotopic homogeneity between skin and muscle was proven, the sampling technique may be the responsible of determining the use of one or the other tissue. For example, the use of skin is important to minimize harm to animals that are sampled while alive. Contrarily, when sampling dead animals, it might be easier to analyse muscle instead of skin, as the first is easily pulverized to obtain an homogeneous dust, while skin, when desiccated, acquires a flexible-plastic consistency difficult to convert in even particles.[41]

Kidney and liver showed statistically higher $\delta^{15}N$ values than skin and muscle for both species; however, $\delta^{15}N$ values did not differ between kidney and liver or between skin and muscle. Borrell et al.[19] reported similar results for fin whale tissues. As well, Caut et al.[10] found that in mammals mean $\delta^{15}N$ values are approximately 0.6‰ higher in the liver than in the muscle.

In mammals, the high $\delta^{15}N$ and $\delta^{13}C$ in the liver compared with the values in other tissues have been partially attributed to the higher metabolic activity of splanchnic organs compared to structural tissues.[16,49,50] Tieszen et al.[16] demonstrated that the carbon turnover rate was linearly correlated with the metabolic rate of tissues. They reported a higher turnover rate for liver than for muscle, brain or hair in laboratory gerbils. Similarly, in a study of quails, Hobson et al.[50] determined a carbon half-life of 2.6 days in liver compared with 173.3 days in bone collagen. The turnover in kidney is

unknown, as it is not typically analysed for stable isotopes. However, its high $\delta^{15}N$ and $\delta^{13}C$ values are in agreement with the generally higher turnover rates of internal organs compared with other tissues such as muscle.[16]

The differences between tissues here described highlight the need to take into account the isotopic differences existing among tissues when comparing results obtained on stable isotope ratios stemming from studies in which different tissues have been used.[51] For example, two species may be incorrectly reported to feed at different trophic levels if different tissues are sampled (e.g. liver and muscle). The differences here determined in stable isotope ratios between tissues constitute the basis for introducing corrections in such situations.

CONCLUSIONS

Isotopic homogeneity within cetacean skin suggests that the isotope ratios of a sample from a certain skin position can be considered representative of the ratios from the entire skin tissue. This conclusion validates the results of previous stable isotope analyses in cetaceans that used skin samples as representative of the whole skin tissue. Muscle and skin provided similar isotopic information and might therefore be used interchangeably for isotope analysis in the current populations, although further analyses should be carried out before drawing general conclusions. Liver and kidney showed higher isotope ratios than muscle and skin. These differences need to be considered when analysing these tissues and comparing the results with those from other tissues obtained from the same or different species.

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Table 1. Biological and sampling data from the animals included in the present study.

Individual code	Species	Sampling date	Gender	Length (cm)
Scoe2008-1	Striped dolphin	11/01/2008	Female	210
Scoe2008-2	Striped dolphin	22/01/2008	Male	194
Scoe2008-6	Striped dolphin	15/04/2008	Female	189
Scoe2008-7	Striped dolphin	21/04/2008	Male	185
Scoe2008-8	Striped dolphin	20/05/2008	Female	190
Scoe2008-9	Striped dolphin	27/02/2008	Male	205
Scoe2008-10	Striped dolphin	30/09/2008	Female	185
Scoe2008-11	Striped dolphin	18/12/2008	Male	204
Scoe2009-1	Striped dolphin	15/03/2009	Male	224
Ddel1	Common dolphin	28/03/2001	Male	187
Ddel2	Common dolphin	11/07/2001	Male	202
Ddel4	Common dolphin	19/07/2001	Female	189
Ddel5	Common dolphin	23/07/2001	Female	179
Ddel3	Common dolphin	23/07/2001	Male	204
Ddel10	Common dolphin	23/07/2001	Male	208
Ddel15	Common dolphin	18/07/2002	Female	206
Ddel16	Common dolphin	18/07/2002	Female	187
Ddel23	Common dolphin	25/07/2002	Female	200
Ddel24	Common dolphin	31/07/2002	Male	201

Table 2. Stable isotope ratios (Mean ± *S.D.*) for each tissue (liver, kidney and muscle) and each skin position for common and striped dolphins.

	Common dolphin				Striped dolphin			
	$\delta^{15}N$ value		$\delta^{13}C$ value		$\delta^{15}N$ value		$\delta^{13}C$ value	
	Mean	***S.D.***	**Mean**	***S.D.***	**Mean**	***S.D.***	**Mean**	***S.D.***
Skin 1	12.37	*0.71*	-17.81	*0.51*	10.23	*0.41*	-17.83	*0.25*
Skin 2	12.31	*0.70*	-17.75	*0.55*	10.41	*0.40*	-17.57	*0.35*
Skin 3	12.39	*0.67*	-17.88	*0.57*	10.41	*0.42*	-18.04	*0.27*
Skin 4	12.44	*0.54*	-17.70	*0.51*	10.39	*0.39*	-17.71	*0.37*
Skin 5	12.36	*0.69*	-17.77	*0.77*	10.39	*0.40*	-17.74	*0.39*
Skin 6	12.48	*0.76*	-17.86	*0.35*	10.42	*0.34*	-17.85	*0.29*
Skin 7	12.32	*0.60*	-17.70	*0.56*	10.40	*0.41*	-17.88	*0.24*
Skin 8	12.36	*0.74*	-17.52	*0.53*	10.34	*0.37*	-17.71	*0.34*
Skin 9	12.47	*0.76*	-17.92	*0.40*	10.41	*0.34*	-17.96	*0.37*
Skin 10	12.28	*0.73*	-17.63	*0.48*	10.35	*0.30*	-17.80	*0.24*
Skin 11	12.41	*0.74*	-17.66	*0.58*	10.46	*0.31*	-17.92	*0.51*
Liver	13.40	*0.62*	-17.56	*0.28*	11.00	*0.5*	-17.51	*0.45*
Kidney	13.30	*0.59*	-17.73	*0.54*	11.18	*0.46*	-17.54	*0.20*
Muscle	12.55	*0.63*	-17.71	*0.45*	10.16	*0.38*	-17.73	*0.33*

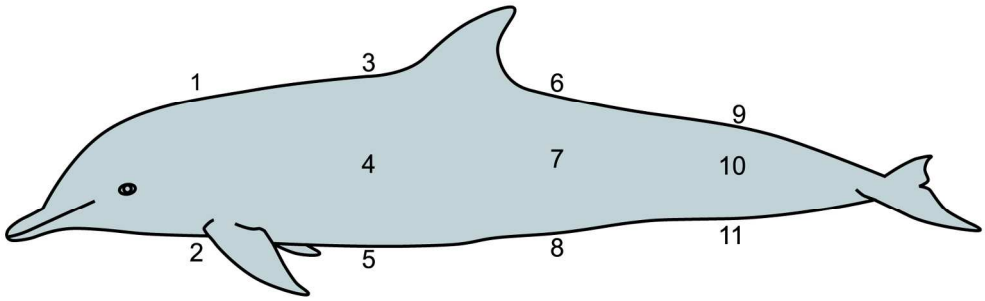

Figure 1. Location within the body of the 11 skin positions (1-11).

166x53mm (300 x 300 DPI)

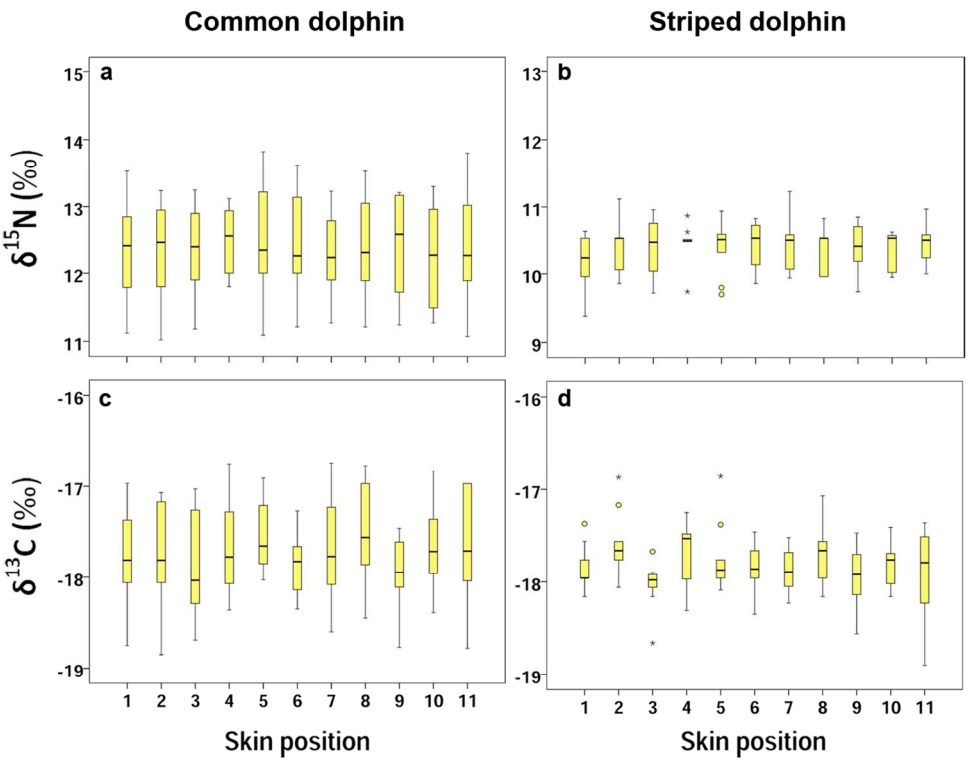

Figure 2. $\delta^{15}N$ (‰) (a, b) and $\delta^{13}C$(‰) (c, d) values in the 11 skin positions sampled for common and striped dolphins.

122x99mm (300 x 300 DPI)

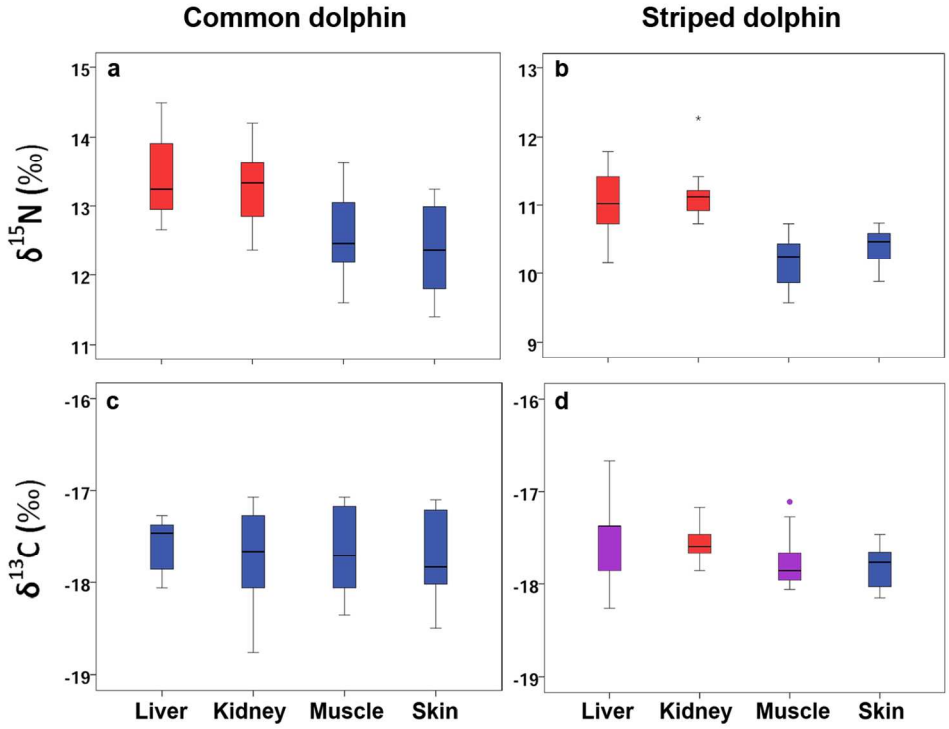

Figure 3. $\delta^{15}N$ (‰) (a, b) and $\delta^{13}C$ (‰) (c, d) values in the four tissues considered for common and striped dolphins. Tissues that significantly differed in isotope ratios are represented by different colours. Tissues that did not significantly differ from those either red or blue are represented in purple.

126x99mm (300 x 300 DPI)